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NAD(P)H:quinone oxidoreductase expression in *Cyp1a*-knockout and *CYP1A*-humanized mouse lines and its effect on bioactivation of the carcinogen aristolochic acid I

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Abbreviations: AHR, aryl hydrocarbon receptor; AA, aristolochic acid; AAI, aristolochic acid I; AAIa, aristolochic acid Ia; AAN, aristolochic acid nephropathy; ARE, antioxidant response element (also known as EpRE, electrophile response element);; BEN, Balkan endemic nephropathy; CYP, cytochrome P450; dA-AAI, 7-deoxyadenosine-N^6-yl)aristolactam I; dA-AAII, 7-deoxyadenosine-N^6-yl)aristolactam II; dG-AAI, 7-deoxyguanosin-N^2-yl)aristolactam I; HRN, Hepatic P450 Reductase Null, KEAP1, Kelch-like ECH-associating protein 1; NATs, *N,O*-acetyltransferases; NQO1, NAD(P)H:quinone oxidoreductase; NRF2, nuclear factor-erythroid-related factor 2; POR, P450 oxidoreductase; RAL, relative adduct labeling; ROS, reactive oxygen species; SDS, sodium dodecyl sulfate; SULTs, sulfotransferases; TLC, thin-layer chromatography; UUC, upper urinary tract urothelial carcinoma; WT, wild-type; XRE, xenobiotic response element (also known as AHRE, AHR response element).

Abstract

Aristolochic acid causes a specific nephropathy (AAN), Balkan endemic nephropathy, and urothelial malignancies. Using Western blotting suitable to determine protein expression, we investigated in several transgenic mouse lines expression of NAD(P)H:quinone oxidoreductase (NQO1)—the most efficient cytosolic enzyme that reductively activates aristolochic acid I (AAI). The mouse tissues used were from previous studies [Arlt *et al., Chem. Res. Toxicol.* 24 (2011) 1710; Stiborova *et al., Toxicol. Sci.* 125 (2012) 345], in which the role of microsomal cytochrome P450 (CYP) enzymes in AAI metabolism *in vivo* had been determined. We found that NQO1 levels in liver, kidney and lung of *Cyp1a1(−/−)*, *Cyp1a2(−/−)* and *Cyp1a1/1a2(−/−)* knockout mouse lines, as well as in two *CYP1A*-humanized mouse lines harboring functional human *CYP1A1* and *CYP1A2* and lacking the mouse *Cyp1a1/1a2* orthologs, differed from NQO1 levels in wild-type mice. NQO1 protein and enzymic activity were induced in hepatic and renal cytosolic fractions isolated from AAI-pretreated mice, compared with those in untreated mice. Furthermore, this increase in hepatic NQO1 enzyme activity was associated with bioactivation of AAI and elevated AAI-DNA adduct levels in *ex vivo* incubations of cytosolic fractions with DNA and AAI. In conclusion, AAI appears to increase its own metabolic activation by inducing NQO1, thereby enhancing its own genotoxic potential.

Keywords: Aristolochic acid nephropathy; Balkan endemic nephropathy; NAD(P):quinone oxidoreductase; Protein expression; Metabolic activation; DNA adducts; Mouse models

INTRODUCTION

The herbal drug aristolochic acid (AA) derived from *Aristolochia* species has been shown to be the cause of so-called Chinese herbs nephropathy, now termed aristolochic acid nephropathy (AAN) (Debelle *et al.*, 2008; Schmeiser *et al.*, 2009). The plant extract AA is a mixture of structurally related nitrophenanthrene carboxylic acids, the major components being aristolochic acid I (AAI) and aristolochic acid II (AAII).

AAN is a rapidly progressive renal fibrosis that was initially observed in a group of Belgian women who had ingested weight loss pills containing *Aristolochia fangchi* (Vanherweghem *et al.*, 1993; Nortier *et al.*, 2000). Within a few years of taking the pills, AAN patients also showed a high risk (~50%) of upper urothelial tract carcinoma and, subsequently, bladder urothelial carcinoma. In the meantime, similar cases have been reported elsewhere in Europe and Asia (Schmeiser *et al.*, 2009). Dietary exposure to AA has also been linked to Balkan endemic nephropathy (BEN) and its associated urothelial cancer (Arlt *et al.*, 2007; Grollman *et al.*, 2007; Moriya *et al.*, 2011); this nephropathy is endemic in certain rural areas of Serbia, Bosnia, Croatia, Bulgaria and Romania.

Exposure to AA was demonstrated by identification of specific AA-DNA adducts in urothelial tissue of AAN and BEN patients (Schmeiser *et al.*, 1996; Nortier *et al.*, 2000; Arlt *et al.*, 2002; Grollman *et al.*, 2007; Jelakovic *et al.*, 2012; Yun *et al.*, 2012). The most abundant DNA adduct detected in patients is 7-(deoxyadenosin-N^6-yl)aristolactam I (dA-AAI) (Fig. 1), which causes characteristic AT→TA transversions. Such AT→TA mutations have been observed in the *TP53* tumor suppressor gene in tumors from AAN and BEN patients (Lord *et al.*, 2004; Grollman *et al.*, 2007; Moriya *et al.*, 2011), indicating a probable molecular mechanism associated with AA-induced carcinogenesis (Arlt *et al.*, 2007; Kucab *et al.*, 2010). More recently, AA exposure was discovered to contribute to the high incidence of upper urinary tract urothelial carcinoma (UUC) in Taiwan, where medicinal use of *Aristolochia* plants is widespread (Chen *et al.*, 2012); again, the *TP53* mutational signature in patients with UUC was predominant among otherwise rare AT→TA transversions (Olivier *et al.*, 2012). AA has been classified as a Group I carcinogen in humans by the International Agency for Research on Cancer (Grosse *et al.*, 2009).

The activation pathway for AAI is nitroreduction, catalyzed by both cytosolic and microsomal enzymes**;** in this process NAD(P)H:quinone oxidoreductase (NQO1) is the most efficient cytosolic nitroreductase (Stiborova *et al.*, 2003; Stiborova *et al.*, 2008a; Stiborova *et*

al., 2008b; Chen *et al.*, 2011) (Fig. 1). In contrast to NQO1, conjugation enzymes such as human sulfotransferases (SULTs) or *N,O*-acetyltransferases (NATs) did not significantly activate AAI (Martinek *et al.*, 2011; Stiborova *et al.*, 2011). In human hepatic microsomes, AAI is activated by cytochrome P450 1A2 (CYP1A2) and, to a lesser extent, by CYP1A1**;** P450 oxidoreductase (POR) also plays a minor role (Stiborova *et al.*, 2001a; Stiborova *et al.*, 2005). Human and rodent CYP1A1 and 1A2 are also the principal enzymes involved in oxidative detoxication of AAI to 8-hydroxyaristolochic acid I (aristolochic acid Ia, AAIa, Fig. 1) (Sistkova *et al.*, 2008; Shibutani *et al.*, 2010).

The role of cytochrome P450 enzymes, particularly CYP1A1 and CYP1A2, both in the reductive activation and oxidative detoxication of AAI, was demonstrated in several animal studies. Two studies used the Hepatic P450 Reductase Null (HRN) mice—in which the *Por* gene is deleted specifically in hepatocytes—resulting in absence of CYP activity (Xiao *et al.*, 2008; Levova *et al.*, 2011), two others used *Cyp1a1(−/−)*, *Cyp1a2(−/−)* and/or *Cyp1a1/1a2(−/−)* mouse lines (Rosenquist *et al.*, 2010; Arlt *et al.*, 2011a).

We also evaluated AAI metabolism mediated by human CYP1A1 and 1A2, employing two *CYP1A*-humanized mouse lines, both carrying functional human *CYP1A1* and *CYP1A2* genes in place of the orthologous mouse genes**;** one line carries the high-affinity aryl hydrocarbon receptor (AHR) [*hCYP1A1_1A2_Cyp1a1/1a2(−/−)_Ahr*^{b1}], whereas the other line carries the poor-affinity AHR [*hCYP1A1_1A2_Cyp1a1/1a2(−/−)_Ahr*^{d}] (Stiborova *et al.*, 2012). The latter line is believed to be more relevant to human risk assessment vis-à-vis human CYP1A1 and CYP1A2 substrates, because poor-affinity, rather than high-affinity, AHR is known to predominate by far in human populations (Nebert *et al.*, 2004). Overall, AAI-DNA adduct levels were higher in *CYP1A*-humanized mice than in wild-type (WT) mice, suggesting strongly that human CYP1A1 and 1A2 causes higher AAI bioactivation than mouse CYP1A1 and CYP1A2 (Stiborova *et al.*, 2012).

Moreover, an exclusive role of human CYP1A1 and 1A2 in AAI oxidation to AAIa was observed in human liver microsomes under aerobic (*i.e.* oxidative) conditions (Stiborova *et al.*, 2012). Our results suggest that, in addition to CYP1A1 and 1A2 expression levels, pO_2 levels in specific organs or even cells might affect the balance between AAI nitroreduction (bioactivation) and demethylation (detoxication) which, in turn, would influence tissue-specific toxicity or carcinogenicity. However, reductive activation of AAI in these mouse lines may not only be dictated by CYP1A1/1A2 (Arlt *et al.*, 2011a; Stiborova *et al.*, 2012) but also by NQO1 expression. Indeed, higher AAI-DNA adduct levels in HRN than in WT mice

are not only the result of lack of hepatic AAI demethylation by the CYP-dependent system, but also by higher NQO1 activity, which activates AAI (Levova *et al.*, 2011). Expression of NQO1 protein in hepatic cytosolic samples of HRN mice was more than 3-fold higher than that in WT mice (Levova *et al.*, 2011); this was paralleled by increased NQO1 activity and AAI-DNA adduct formation in *ex vivo* cytosolic incubations with DNA and AAI. Collectively, these results suggest that deletion of the *Por* gene and absence of the POR enzyme in liver is partially compensated by increases in expression of the cytosolic nitroreductase NQO1 (Levova *et al.*, 2011).

In the present study, the influence of AAI exposure upon NQO1 protein expression in *Cyp1a*-knockout and *CYP1A*-humanized mouse lines was studied by Western blot. Hepatic, renal and pulmonary cytosolic NQO1 expression in untreated vs AAI-pretreated mice was evaluated. NQO1 enzyme activity plus AAI-DNA adduct formation catalyzed by hepatic cytosols were also determined.

MATERIAL AND METHODS

Animal experiments. Generation of *Cyp1a1(−/−)* (Dalton et al., 2000), *Cyp1a2(−/−)* (Liang et al., 1996), and *Cyp1a1/1a2(−/−)* (Dragin et al., 2007) knockout mouse lines (on a >99.8% C57BL/6J background) has been described. Creation of *CYP1A*-humanized mouse lines—namely, *hCYP1A1_1A2_Cyp1a1/1a2(−/−)_Ahr*^{b1} and *hCYP1A1_1A2_Cyp1a1/1a2(−/−)_Ahr*^{d} (both on a >99.8% C57BL/6J background—has also been described (Dragin et al., 2007; Shi et al., 2008). Age-matched C57BL/6J *Cyp1(+/+)* WT mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). Groups of female mice (3 months old; 25-30 g; n = 4/group) were treated with a single dose of AAI as sodium salt in water (50 mg/kg body weight) by oral gavage 24 h before sacrifice (Arlt et al., 2011a; Stiborova et al., 2012). All experiments were approved by, and conducted in accordance with, the National Institute of Health standards for the care and use of experimental animals and the University of Cincinnati Medical Center Institutional Animal Care and Use Committee.

Preparation of cytosolic samples. Hepatic, renal and pulmonary cytosolic fractions from untreated and AAI-pretreated *Cyp1a1(−/−)*, *Cyp1a2(−/−)*, and *Cyp1a1/1a2(−/−)* mice (Arlt *et al.*, 2011a), *CYP1A*-humanized (Stiborova *et al.*, 2012) and WT mice (Arlt *et al.*, 2011a; Stiborova *et al.*, 2012) were isolated as previously described (Stiborova *et al.*, 2003; Martinek *et al.*, 2011; Stiborova *et al.*, 2011). Pooled cytosolic fractions (*n* = 4 mice/group) were used for these analyses.

Determination of NQO1 protein levels by Western blotting. NQO1 antibodies were prepared as described previously (Stiborova *et al.*, 2006). Immunoquantification of cytosolic NQO1 was carried out on proteins transferred to nitrocellulose membranes after separation by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (Stiborova *et al.*, 2006). Human recombinant NQO1 (Sigma) was used to identify the NQO1 band from mouse cytosol. Glyceraldehyde phosphate dehydrogenase was used as loading control and detected by its antibody (1:750, Millipore; MA, USA).

Measurement of NQO1 enzyme activity. NQO1 activity was determined using menadione as substrate as reported, a method improved by the addition of cytochrome *c* (Mizerovska *et al.*, 2011).

Cytosolic AAI-DNA adduct formation by ^{32}P-postlabeling. Incubation mixtures, in a final volume of 750 µl, included 50 mM Tris-HCl buffer (pH 7.4) containing 0.2% Tween 20, 1 mM NADPH, 1 mg mouse liver cytosolic protein, 0.5 mg calf thymus DNA (2 mM dNp) and 0.5 mM AAI (Stiborova *et al.*, 2003). Incubations were carried out at 37°C for 60 min; AAI-derived DNA adduct formation is known to be linear up to 2 hr (Stiborova *et al.*, 2003). Control incubations were carried out (*i*) without cytosol, (*ii*) without NADPH, (*iii*) without DNA, or (*iv*) without AAI. After extraction with ethyl acetate, DNA was isolated from the residual water phase by the standard phenol/chloroform extraction method. ^{32}P-Postlabelling assay using the nuclease P1 enrichment version, and thin-layer chromatography (TLC) for analysis of AAI-DNA adduct formation, were performed as described (Schmeiser *et al.*, 1996; Bieler *et al.*, 1997). TLC sheets were scanned using a Packard Instant Imager (Dowers Grove; USA). DNA adduct levels (RAL, relative adduct labeling) were calculated as described (Schmeiser *et al.*, 1996; Stiborova *et al.*, 2003). Results were expressed as DNA adducts/10^8 nucleotides. Because of low protein concentrations in renal and pulmonary cytosolic fractions, AAI-DNA adduct formation in these cytosolic samples was not carried out.

RESULTS

Using the Western blot method, we compared NQO1 protein levels in *Cyp1a*-knockout, *CYP1A*-humanized mouse lines, and WT mice. In addition, the effect of AAI treatment was also examined.

NQO1 protein levels and enzyme activity in Cyp1a-knockout mouse lines

NQO1 protein was detected in all cytosolic samples investigated. Statistically significantly higher levels of NQO1 expression were found in liver, kidney and lung of untreated *Cyp1a1(−/−)*, *Cyp1a2(−/−)* and *Cyp1a1/1a2(−/−)* mice, compared with those in WT mice (Fig. 2). Higher NQO1 protein levels in liver of all *Cyp1a*-knock-out mice paralleled higher NQO1 enzyme activities (Fig. 2, left panel). However, this was not the case in kidney: NQO1 enzyme activity was not different among *Cyp1a1(−/−)*, *Cyp1a2(−/−)* and WT mice and was lower in *Cyp1a1/1a2(−/−)* mice (Fig. 2, middle panel). NQO1 protein levels in lung could not be compared with NQO1 enzyme activity, because NQO1 activity was not detectable in *Cyp1a1(−/−)* or *Cyp1a1/1a2(−/−)* lung cytosol (Fig. 2, right panel)—despite lung NQO1 protein levels that appeared similar to levels seen in liver. AAI pretreatment induced NQO1 protein levels not only in WT liver, kidney and lung cytosol but also in *Cyp1a1(−/−)* liver, Cyp1a1(−/−) kidney, *Cyp1a2(−/−)* kidney, *Cyp1a1/1a2(−/−)* kidney, *Cyp1a1(−/−)* lung, and *Cyp1a2(−/−)* lung (Fig. 2). This increase was associated with enhanced NQO1 enzyme activities in liver and kidney cytosolic fractions (Fig. 2, lower panels). No NQO1 enzyme activity was detectable in pulmonary cytosol from AAI-pretreated mice.

NQO1 protein levels and enzyme activity in CYP1A-humanized mouse lines

Liver and kidney NQO1 protein levels were significantly higher in both lines of *CYP1A*-humanized mice, compared with those in WT mice. In lung cytosol from *hCYP1A1_1A2_Cyp1a1/1a2(−/−)_Ahr*^{d}, lower NQO1 protein levels were found (Fig. 3). Overall, NQO1 enzyme activity paralleled protein levels in liver and kidney**;** however, no cytosolic NQO1 activity was detectable in lung of either CYP1A-humanized mouse line (Fig. 3).

AAI pretreatment enhanced cytosolic NQO1 protein levels also in *CYP1A*-humanized mice**:** 2.6- and 2.3-fold higher, respectively, in liver from *hCYP1A1_1A2_Cyp1a1/1a2(−/−)_Ahr*^{b1} and *hCYP1A1_1A2_Cyp1a1/1a2(−/−)_Ahr*^{d}, compared with that in untreated mice. Also in kidney from *hCYP1A1_1A2_Cyp1a1/1a2(−/−)_Ahr*^{b1} mice, 1.4-higher NQO1 protein levels, than in untreated mice were found (Fig. 3).

Increased renal NQO1 protein levels were associated with enhanced NQO1 enzyme activity (Fig. 3, *middle panel*). Cytosolic NQO1 enzyme activity was either the same or lower in liver from AAI-pretreated *CYP1A*-humanized mice than that of untreated mice (Fig. 3, *left panel*).

More than 2-fold higher NQO1 protein levels were also induced by AAI in lung cytosol in *hCYP1A1_1A2_Cyp1a1/1a2(−/−)_Ahr*^{b1} and *hCYP1A1_1A2_Cyp1a1/1a2(−/−)_Ahr*^{d} mice relative to those of untreated mice, but no cytosolic NQO1 enzyme activity was detectable in the lung of either *CYP1A*-humanized mouse line (Fig. 3, *right panel*).

AAI-DNA adduct formation mediated by liver cytosol

Hepatic cytosol from AAI-pretreated mice was compared with that from untreated mice (Table 1). AAI was metabolically activated in all samples tested, to generate the same pattern of AAI-DNA adducts, consisting of three DNA adducts (see Fig. 1, insert) determined by ^{32}P-postlabeling. This same DNA adduct pattern has been observed in urothelial tissue from AAN and BEN patients (Nortier et al., 2000; Arlt et al., 2002). These adducts have previously been identified (Schmeiser et al., 1996; Stiborova et al., 2003) as 7-(deoxyadenosin-N^6-yl)aristolactam I (dA-AAI), 7-(deoxyguanosin-N^2-yl)aristolactam I (dG-AAI) and 7-(deoxyadenosin-N^6-yl)aristolactam II (dA-AAII) (Fig. 1). No DNA adducts were observed in control incubations carried out in parallel without cytosol, or without DNA, or without AAI (data not shown).

Cyp1a1(−/−), Cyp1a2(−/−) and *Cyp1a1/1a2(−/−)* hepatic cytosol from untreated mice led to as much as ~2-fold higher AAI-DNA adduct levels than that from WT mice (Table 1). Likewise, hepatic cytosol from untreated *hCYP1A1_1A2_Cyp1a1/1a2*(−/−)*_Ahr*^{d} mice catalyzed ~2-fold higher AAI-DNA adduct levels than that from WT mice, whereas no statistically significantly different adduct levels were seen with

hCYP1A1_1A2_Cyp1a1/1a2(−/−)*_Ahr*^{b1} cytosol. Higher AAI-DNA adducts were only observed with cytosols from these mice after AAI treatment, when all cytosols exhibited similar AAI activating potency – irrespective of their genotype (Table 1). These enhanced AAI-DNA adduct levels corresponded to higher cytosolic NQO1 enzyme activity (compare Figs. 2 & 3).

DISCUSSION

Previously we found that AAI, in the presence of NADPH (a cofactor of NQO1), is activated by human liver and kidney cytosolic fractions, as well as by purified human NQO1, to DNA adducts identical to those found in humans diagnosed with AAN and BEN (Stiborova *et al.*, 2003; Martinek *et al.*, 2011; Stiborova *et al.*, 2011). These data suggested that NQO1 might be the principle enzyme responsible for AAI activation. In the present study, we used Western blotting analysis to determine NQO1 protein levels in cytosol from liver, kidney and lung of *Cyp1a*-knockout mice lacking the CYP1A1 and/or CYP1A2 enzymes, and in *CYP1A*-humanized mouse lines expressing human CYP1A1 and 1A2 in place of the mouse orthologs. NQO1 enzyme activity and AAI-DNA adduct levels formed in *in-vitro* incubations were also measured. Further, we evaluated not only basal NQO1 expression, but also NQO1 expression following AAI pretreatment. These data should enhance our understanding of the potential role of NQO1 in AAI bioactivation.

Our results demonstrate that cytosolic NQO1 protein levels and enzyme activity are higher in liver and kidney from the three *Cyp1a*-knockout mouse lines and the *CYP1A*-humanized mice**;** NQO1 activity in lung was not detected, despite measurable protein levels. Higher liver cytosolic NQO1 activity resulted in bioactivation and, hence, elevated AAI-DNA adduct formation, when cytosol was incubated *ex vivo* with DNA and AAI. The highest DNA adduct levels were seen with cytosol from *Cyp1a2(−/−)* and *Cyp1a1/1a2(−/−)* as well as from *hCYP1A1_1A2_Cyp1a1/1a2(−/−)_Ahr*^{d} mice. In contrast, only slightly higher AAI-DNA adduct levels were generated by *Cyp1a1(−/−)* hepatic cytosol. This finding is consistent with a previous study showing that ablation of the *Cyp1a1* gene was not sufficient to alter hepatic constitutive expression of other genes in the mouse Ahr battery including *Nqo1* (Dalton *et al.*, 2000).

Our results also indicate that pretreatment with AAI induces NQO1 protein levels and enzyme activity in liver and kidney cytosol from these transgenic as well as WT mice. The highest increase in NQO1 was found in kidney, followed by lung and liver. These data suggest that AAI can induce NQO1 in kidney – which is the target organ of AAI-induced toxicity; moreover, AAI induced NQO1 in liver, the most important organ responsible for metabolism of xenobiotics including AAI. This is consistent with previous studies where NQO1 activity was increased in kidney of rats pretreated with AAI (Stiborova *et al.*, 2001b), and NQO1 protein levels were induced by AAI in mouse kidney (Arlt *et al.*, 2011b). Hence,

this enzyme is likely to be induced in the kidneys of AAN and BEN patients, which may contribute to their increased risk for urothelial cancer.

Thus far, we can only speculate on the mechanisms responsible for AAI-mediated NQO1 induction. NQO1 expression has been shown to be regulated by two distinct regulatory elements in the 5' flanking region of the *NQO1* gene, the antioxidant response element (ARE) and the xenobiotic response element (XRE), involving ligand-activated AHR (Nebert and Jones, 1989; Ross *et al*., 2000; Jaiswal, 2004; Nioi and Hayes, 2004). In ARE-mediated *NQO1* induction, nuclear factor-erythroid 2-related factor 2 (NRF2) and the cytoskeletal-binding protein KEAP1 (Kelch-like ECH-associating protein 1) play an important role (*i.e.* the NRF2-KEAP1 mechanism of NQO1 induction). ARE-mediated *NQO1* gene expression is increased by a variety of antioxidants, tumor promoters and reactive oxygen species (ROS) (Li and Jaiswal, 1994). ROS has been shown to be generated in several human cell lines in culture after AAI exposure (Yu *et al.,* 2011; Zhu *et al.,* 2012). Hence, an increase in AAI-mediated ROS formation might be one mechanism by which AAI induces NQO1. However, the question whether ROS formation during AAI metabolism is the means by which NQO1 is induced, or by other mechanisms, remains to be explored in future studies.

The XRE-mediated *NQO1* induction is still a matter of debate. The human XRE of *NQO1* shares significant homology with the human CYP1A1 XRE (Nebert *et al.,* 2000). Both *NQO1* and *CYP1A1* genes can be induced by AHR ligands such as 2,3,7,8-tetrachlorodibenzo[1,4]dioxine and polycyclic aromatic hydrocarbons (Nebert *et al.,* 2000; Ross *et al.,* 2000; Nioi and Hayes, 2004). However, some AHR ligands such as β-naphthoflavone or certain azo dyes can be metabolized by CYPs to oxidative metabolites that can activate ARE-driven gene expression (De Long *et al.,* 1987). Prochaska and Talalay (1988) have referred to compounds that can stimulate both XRE- and ARE-driven gene expressions as "bi-functional inducing agents". Moreover, cross-talk may occur between NRF2 and AHR, but the details of this process remain to be elucidated (Jaiswal, 2004). We found that AAI treatment led to an increase in ethoxyresorufin *O*-deethylation, methoxyresorufin *O*-demethylation and Sudan I oxidation in mice (Arlt *et al.,* 2011a; Stiborova *et al.,* 2012), all of which are indicators of the XRE-regulated enzymes CYP1A1 and 1A2. Thus, AAI-mediated enzyme induction by XRE-regulated pathways should also be taken into account. Determining which of the above mentioned two mechanism(s) is(are)

responsible for AAI-mediated induction of NQO1 and CYP1A will be one of the major challenges for future research.

Of note, it was reported that polymorphisms in the human *NQO1* gene are important in AA-induced nephropathy (Toncheva *et al.*, 2004; Toncheva, 2006;). Indeed, the *NQO1*2/*2* genotype, resulting in very low NQO1 levels of expression, was shown to predispose BEN patients to a much higher incidence of urothelial cancer (OR=13.75, 95%CI 1.17-166.21) (Toncheva *et al.*, 2004; Toncheva, 2006). This finding appears to be opposite to what one might expect, given our demonstration herein of the importance of NQO1 in AAI activation; however, diminished NQO1 metabolism of AAI could lead to an enhanced body burden which might lead to increased risk of tumorigenesis over time.

The situation in lung is completely different from that in the other two organs investigated. Basal and induced NQO1 protein levels in lung, as measured by Western blotting was similar to that in liver or kidney, but NQO1 enzyme activity was much lower or not detectable in lung, especially following AAI pretreatment. Several reasons for this observation are possible**:** the protein that is expressed is in an inactive form; an inhibitor might be present in lung cytosol; menadione is not a good substrate for lung NQO1; or the lung NQO1 protein has undergone allosteric effects. Which of these might be most important in our experiments is hard to say, but it was noticeable that in all cases the lung NQO1 enzyme activity was not seen following AAI pretreatment.

In the present study, increases in NQO1 protein levels in kidney and liver correlated with NQO1 enzyme activity in these two organs. In liver cytosol, increases in NQO1 protein levels also paralleled elevated levels of AAI-DNA adducts. Presence of the human CYP1A orthologs regulated by the poor-affinity AHR led to similar results in NQO1 protein levels and enzyme activity as seen in the *Cyp1a*-knock-out mice. Only the high-affinity-AHR humanized line showed NQO1 protein and enzyme activity levels comparable to those seen in wild-type mice. Together with previous studies (Arlt *et al.*, 2011a; Levova *et al.*, 2011; Stiborova *et al.*, 2012), the results herein indicate that, if hepatic CYP1A expressions were missing, leading to less detoxication of AAI by demethylation (as seen in *Cyp1a*-knockout mice), then NQO1 would become the major enzyme, leading to higher AAI-DNA adduct formation. In addition, we have shown that AAI pretreatment can induce NQO1 in liver and kidney, which results in enhanced reductive AAI bio-activation to form hepatic AAI-DNA adducts, thereby enhancing the genotoxic potential of AAI.

In summary, utilizing Western blot analysis, NQO1 protein levels were analyzed in *Cyp1a*-knock-out and *CYP1A*-humanized mouse lines. Deletion of both *Cyp1a* genes in these

mouse lines resulted in elevated expression of NQO1 protein in the liver and kidney. Likewise, *CYP1A*-humanized mouse lines displayed higher levels of the NQO1 protein in liver and kidney. Our results indicate that AAI has the potential to induce the cytosolic NQO1 nitroreductase activity in mouse liver and kidney. Our studies with *CYP1A*-humanized mice – and the findings of others (Toncheva, 2006) that certain *NQO1* genotypes appear to be associated with increased risk of urothelial cancer in BEN patients – underscore the clinical importance of NQO1 activity in humans exposed to AAI. It is possible that these findings can be extrapolated to other nitro-aromatic carcinogens.

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Conflict of interest statement

None.

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Table 1. AAI-DNA adduct formation by hepatic cytosol isolated from *Cyp1a*-knockout, *CYP1A*-humanized and WT mice – untreated *versus* pretreated for 24 h with AAI (single oral dose, 50 mg/kg body weight)

Mouse line	RAL per 10^8 nucleotides	
	untreated mice	mice pretreated with 50 mg/kg body weight AAI
WT	1.9 ± 0.2	3.4 ± 0.3$^{\#\#}$
Cyp1a1(–/–)	2.8 ± 0.3*	3.7 ± 0.4$^{\#}$
Cyp1a2(–/–)	3.7 ± 0.4**	3.1 ± 0.3
Cyp1a1/1a2(–/–)	3.3 ± 0.3**	3.6 ± 0.3
hCYP1A1_1A2_Cyp1a1/1a2(–/–)*_Ahr*^{b1}	2.3 ± 0.2	3.6 ± 0.4$^{\#\#}$
hCYP1A1_1A2_Cyp1a1/1a2(–/–)*_Ahr*^{d}	3.9 ± 0.4**	3.4 ± 0.3

Relative adduct labelling (RAL) values are given as means ± S.E.M. (n = 3). * P<0.05, ** P<0.01 (different from WT untreated mice); $^{\#}$ *p<0.05*, $^{\#\#}$ *p<0.01* (different from mice with the same genetic background not pretreated with AAI. Comparisons were made by Student's *t*-test.

Legends to figures

Figure 1

Pathways of biotransformation and DNA adduct formation of AAI. dA-AAI, 7-(deoxyadenosin-N^6-yl)aristolactam I; dG-AAI, 7-(deoxyguanosin-N^2-yl)aristolactam I; NQO1, NAD(P)H:quinone oxidoreductase; NR, nitro-reductase. Insert: Autoradiographic profile of AAI-DNA adducts formed by incubation of AAI with hepatic cytosol from WT mice, using the nuclease P1 enrichment version of the ^{32}P-postlabeling assay. The adduct profile shown is representative of those obtained in hepatic cytosolic fractions of other mouse lines.

Figure 2

NQO1 protein levels (*black columns*) and NQO1 enzyme activity (*hatched columns*) in cytosol isolated from liver, kidney and lung of WT, *Cyp1a1(−/−)*, *Cyp1a2(−/−)* and *Cyp1a1/1a2(−/−)* mice; animals were either untreated or pretreated with a single AAI oral dose (50 mg/kg body weight). Inserts (*at top*) show Western blots. Cytosol isolated from one organ was analyzed in the same blot (*e.g.* liver untreated *versus* AAI-pretreated mice) and therefore can be directly compared. Human recombinant NQO1 (Sigma) was used to identify the mouse NQO1 band in mouse cytosol (data not shown). All values are given as means ± S.E.M. (n = 3). Values significantly different from WT mice: *$p<0.05$, **$p<0.01$ *and* ***$p<0.001$ (Student's t-test).

Figure 3

Cytosolic NQO1 protein levels (*black columns*) and NQO1 enzyme activities (*hatched columns*) in liver, kidney and lung of WT, *hCYP1A1_1A2_Cyp1a1/1a2(−/−)_Ahr^{b1}* and *hCYP1A1_1A2_Cyp1a1/1a2(−/−)_Ahr^{d}* mice; animals were either untreated or pretreated with oral AAI (50 mg/kg body weight). All values are given as means ± S.E.M. (n = 3). Values significantly different from WT mice: *$p<0.05$, **$p<0.01$ *and* ***$p<0.001$ (Student's t-test).

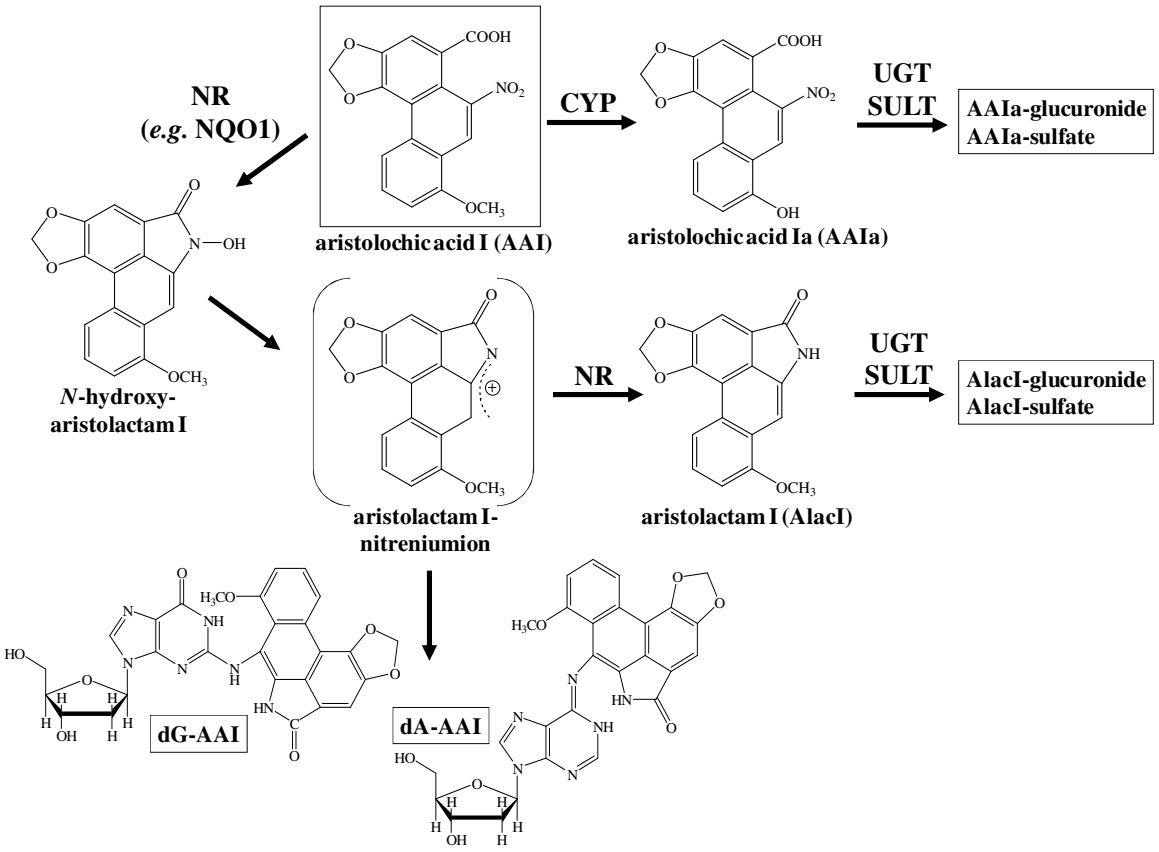
NR
(e.g. NQO1)
aristolochic acid I (AAI)
CYP
aristolochic acid Ia (AAIa)
UGT
SULT
AAIa-glucuronide
AAIa-sulfate
N-hydroxy-
aristolactam I
NR
aristolactam I-
nitreniumion
aristolactam I (AlacI)
UGT
SULT
AlacI-glucuronide
AlacI-sulfate
dG-AAI
dA-AAI

Figure 1

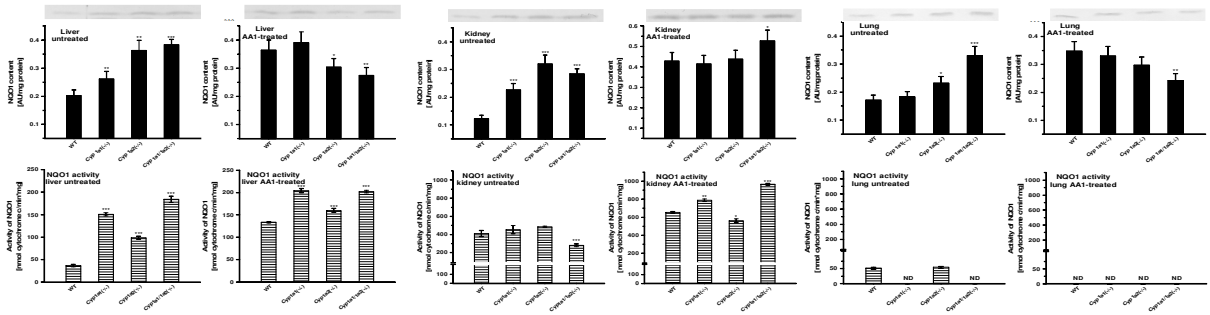
Liver
untreated
Liver
AA1-treated
Kidney
untreated
Kidney
AA1-treated
Lung
untreated
Lung
AA1-treated
NQO1 content
[AU/mg protein]
NQO1 activity
liver untreated
NQO1 activity
liver AA1-treated
NQO1 activity
kidney untreated
NQO1 activity
kidney AA1-treated
NQO1 activity
lung untreated
NQO1 activity
lung AA1-treated
Activity of NQO1
[nmol cytochrome c/min/mg]
ND

Figure 2

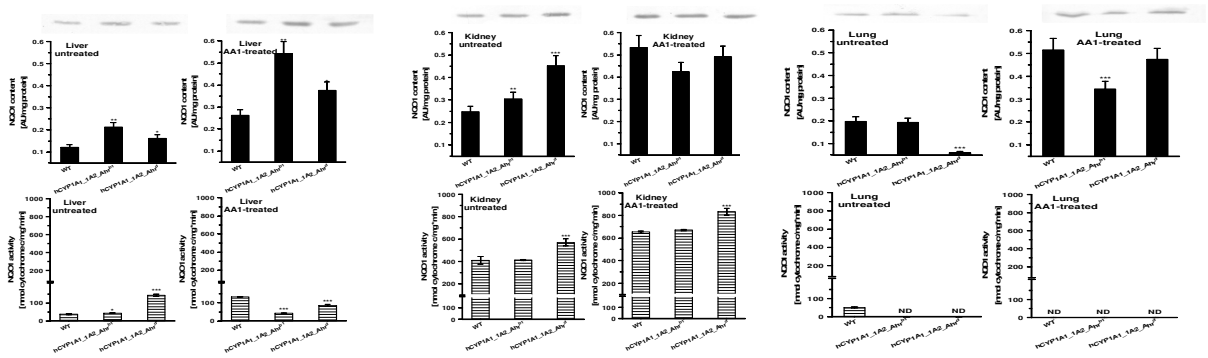
Liver
untreated
Liver
AA1-treated
Kidney
untreated
Kidney
AA1-treated
Lung
untreated
Lung
AA1-treated
NQO1 content
[AU/mg protein]
NQO1 activity
[nmol cytochrome c/mg*min]
WT
hCYP1A1_1A2_Ahr-/-
hCYP1A1_1A2_Ahr+/+
ND

Figure 3